[CANCER RESEARCH 41, 937-941, March 1981]

Uptake of Hematin and Growth of Malignant Murine Erythroleukemia Cells Depleted of Endogenous Heme by Succinylacetone

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ABSTRACT

Heme levels and growth of malignant murine erythroleukemia cells in heme-free medium are drastically reduced by incubation of these cells in the presence of 4,6-dioxoheptanoic acid [succinylacetone (SA)]. When hematin was added to the culture medium of heme-depleted cells, the intracellular heme levels returned to normal, and growth inhibition produced by SA was also reversed. However, when cells depleted of heme by growth in heme-free medium containing SA were placed in heme-free medium without SA, heme levels were restored to normal, and growth was resumed. Hematin uptake in both untreated and heme-depleted malignant murine erythroleukemia cells exhibited biphasic kinetics, with a rapid phase of about 2 min followed by a slower uptake. The rate of uptake of exogenous hematin was slightly greater at 37° than at 20°. Although supplementation of heme-free medium with exogenous hematin increased total cellular heme in both untreated and heme-depleted malignant murine erythroleukemia cells, the fraction of heme in the 20,000 × *g* sediment was unaffected. A nonmalignant fibroblastic cell line, 3T3, exhibited little or no capacity to take up exogenous hematin.

INTRODUCTION

It has been shown recently that SA[1] can profoundly reduce heme concentrations and inhibit growth of MEL cells by selectively inhibiting the second enzyme of the heme-biosynthetic pathway, δ-aminolevulinic acid dehydratase (3, 13). Inhibition of heme biosynthesis caused intracellular heme concentrations to decline sequentially with each cell division. Cell death usually began after 2 divisions. Growth inhibition could be reversed by the addition of hematin to the heme-free medium which contained gelatin (in place of heme-containing FBS) as the only protein source (13). Addition of exogenous hematin caused an increase of cellular heme concentrations which was accompanied by a resumption of growth, indicating that the cells were able to take up the exogenous hematin and utilize it in the absence of a functional heme pathway. The present study was undertaken to examine further details of the heme uptake in MEL cells and the relationship of this uptake to the reversal of growth inhibition caused by SA.

MATERIALS AND METHODS

Chemicals. SA was obtained from Proteochem, Inc. (Denver, Colo.).

Cells and Culture Procedures. MEL cells from clone 745 were routinely maintained in Roswell Park Memorial Institute Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) containing 10% heat-inactivated FBS as described previously (3). Following a 2-day conditioning period in 1% FBS, the cells were centrifuged and resuspended in Roswell Park Memorial Institute Medium 1640 containing 1% gelatin (Difco Laboratories, Inc., Detroit, Mich.) and 30 μM insulin (13). Viable cells were determined in a hemacytometer by the trypan blue exclusion method. 3T3 fibroblasts were obtained from Flow Labs (Falls Church, Va.) and cloned. The cells were maintained in McCoy's Medium 5A containing penicillin:streptomycin:neomycin (10:10:20 μg/ml) and 10% heat-inactivated FBS. In experiments in which the cells were depleted of heme by SA, the FBS concentration was reduced to 4%. Following incubations of 3T3 cells with hematin, the cells were harvested by either trypsinization or scraping prior to washing 3 times with 10 mM Tris (pH 7.4):0.15 M NaCl.

Heme Determination. Heme was determined by the oxalic acid-fluorometric procedure of Sassa *et al.* (12) with minor modifications. Cells were washed 3 times with 10 mM Tris (pH 7.4):0.15 M NaCl, suspended in 0.5 ml water, and frozen prior to heme determination. Heme specific activity was based on the cellular protein concentration which was measured by the procedure of Lowry *et al.* (9).

Preparation of Hematin. Hematin was prepared from human RBC by the method of Fischer (5). It was recrystallized twice and then dissolved in 0.25% sodium carbonate. The pH was adjusted to 8.0 with N HCl. This solution was passed through a Millipore filter, and aliquots of the sterilized solution were then diluted with 10 mM Tris (pH 7.4):0.15 M NaCl to give the appropriate final concentration of hematin used in the various experiments.

Cell Fractionation. To determine the sites of accumulation of hematin in MEL cells exposed to exogenous hematin for brief periods, the cells were washed 3 times with 10 mM Tris (pH 7.4):0.15 M NaCl, suspended in 1.0 ml water, and then freeze-thawed 5 times. A particulate fraction was obtained by centrifugation at 20,000 × *g* for 20 min, resuspended in water, and frozen prior to heme determination. The supernatant fraction resulting from the centrifugation was likewise frozen.

RESULTS

The long-term effect of hematin addition on heme-depleted and untreated MEL cells is shown in Chart 1*A*, which shows that control cells grew continuously throughout the 10-day experimental period. The addition of hematin at 2 nmol/ml to the heme-free medium did not enhance the growth rate. When 0.3 mM SA was present, there was some growth observed over the first 3 days, although this was markedly inhibited below control values, and no growth occurred thereafter. Cells receiving hematin at 100 pmol/ml in addition to the 0.3 mM SA showed a growth pattern identical to that of 0.3 mM SA alone.

[1] The abbreviations used are: SA, succinylacetone (4,6-dioxoheptanoic acid); MEL, murine erythroleukemia; FBS, fetal bovine serum.

Received July 25, 1980; accepted December 8, 1980.

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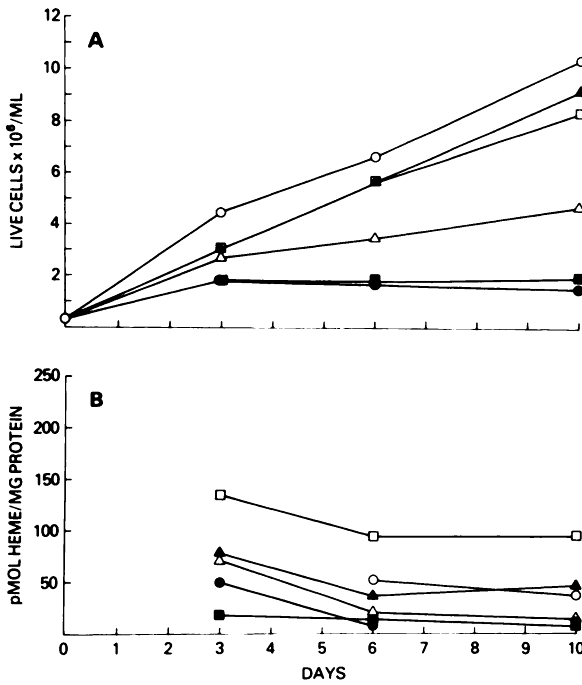

Chart 1. Growth characteristics and heme concentrations in MEL cells grown in defined medium with and without SA and with and without hematin. MEL cells were grown in Roswell Park Memorial Institute Medium 1640 containing 1% gelatin. Growth curves beyond Day 3 are corrected to show cumulative growth. ○, control; ●, 0.3 mM SA; □, hematin (2 nmol/ml); ■, 0.3 mM SA + hematin (100 pmol/ml); △, 0.3 mM SA + hematin (500 pmol/ml); ▲, 0.3 mM SA + hematin (2 nmol/ml). *A*, growth characteristics of MEL cells grown in medium containing SA with and without 3 levels of hematin; *B*, heme concentrations of these cells from Days 3 to 10. Similar results were obtained in 2 additional experiments.

When the concentration of hematin was raised to 500 pmol/ml in the presence of 0.3 mM SA, partial reversal of the growth inhibition produced by SA alone was seen. A concentration of 2 nmol hematin per ml reversed almost completely the growth inhibition produced by SA alone.

The heme content of cells from this experiment is presented in Chart 1*B*. The highest concentrations were seen in cells grown in medium supplemented with the highest concentration of hematin (2 nmol/ml) used in these studies. These levels exceeded those of untreated cells. In general (with the exception of Day 3), supplementation of the growth medium with increasing concentrations of hematin in the presence of 0.3 mM SA caused increasing cellular heme concentrations. Furthermore, there was a general correlation between increasing hematin concentrations in the medium and increasing growth rates of MEL cells exposed to 0.3 mM SA as described in Chart 1*A*.

In Chart 2, cells were exposed to SA at concentrations of 0.3 or 1.0 mM for 4 days after which SA-containing medium was replaced with either fresh medium without SA or fresh medium without SA supplemented with hematin at either 300 pmol/ml or 2 nmol/ml. In *A*, it is seen that continuous exposure to SA at either concentration maintained the profound growth-inhibitory effect of SA, but replacement of medium containing SA with medium from which SA was absent allowed growth to resume. The resumption of growth was most rapid in cells which had been exposed to the lower concentration of SA (0.3 mM) whether the medium was supplemented with hematin or not. Addition of hematin at a concentration of 2 nmol/ml to the medium of cells which had been exposed to 1 mM SA stimulated growth considerably beyond that seen when these cells were not supplemented with hematin and without SA. However, hematin supplementation of the medium of cells previously exposed to 1 mM SA could not restore growth rates to those of cells previously exposed to 0.3 mM SA whether the latter were or were not stimulated by hematin supplementation. Thus, restoration of growth rate was more rapid under all conditions in cells exposed to 0.3 mM SA than those exposed to 1 mM SA.

In Chart 2*B*, the heme concentration is presented for the cells whose growth curves are shown in *A*. When cells were continuously exposed to 0.3 mM SA, a profound decrease in cellular heme concentration occurred. When medium containing SA was replaced with fresh medium without SA on Day 4, cellular heme concentration increased to levels above those of

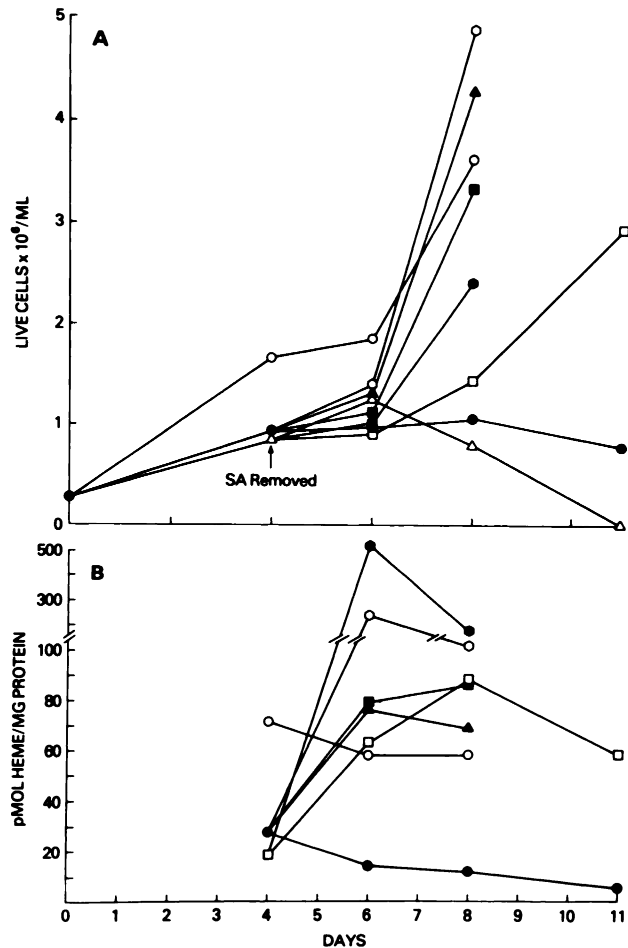

Chart 2. The long-term growth characteristics and heme concentrations of MEL cells grown in defined medium with and without SA up to Day 4; after Day 4, cells were grown with and without SA and supplemented with hematin. MEL cells were grown in Roswell Park Memorial Institute Medium 1640 containing 1% gelatin. When appropriate, the cells were diluted in fresh medium on Day 4, and either SA or hematin was added as indicated. Growth curves beyond Day 4 are corrected to show cumulative growth. ○, control; ●, 0.3 mM SA present continuously; △, 1.0 mM SA present continuously; ▲, 0.3 mM SA to Day 4, then fresh medium; □, 1.0 mM SA to Day 4, then fresh medium; ■, 0.3 mM SA to Day 4, then fresh medium containing hematin (300 pmol/ml); ⬡, 0.3 mM SA to Day 4, then fresh medium containing hematin (2 nmol/ml); ⬢, 1.0 mM SA to Day 4, then fresh medium containing hematin (2 nmol/ml). *A*, growth pattern of MEL cells after the removal of SA on Day 4 and the subsequent addition of SA or hematin; *B*, cellular heme concentrations on Days 4 to 11. Similar results were obtained in 2 additional experiments.

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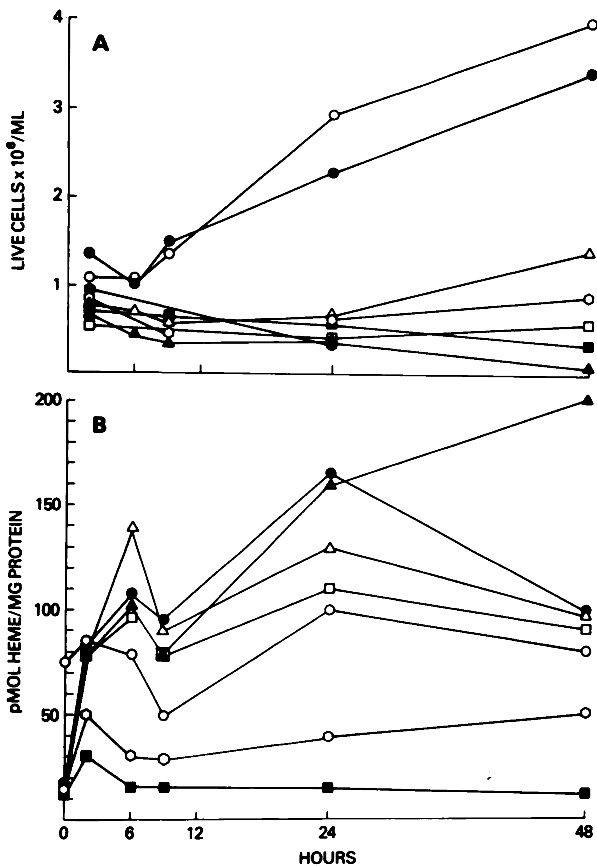

Chart 3. The intermediate-term growth characteristics and heme concentrations of MEL cells grown in defined medium with and without hematin or SA. MEL cells were grown in Roswell Park Memorial Institute Medium 1640 containing 1% gelatin with and without SA for 4 days. The medium was changed, and the cells were incubated further with hematin, fresh medium only, or SA for an additional 48 hr. ○, control; ●, medium only for 4 days, then hematin (500 pmol/ml); △, 0.1 mM SA for 4 days, then hematin (500 pmol/ml); ▲, 0.3 mM SA for 4 days, then hematin (500 pmol/ml); □, 0.6 mM SA for 4 days, then hematin (500 pmol/ml); ■, 0.3 mM SA for 6 days; ⬡, 0.3 mM SA for 4 days, then medium only; [illegible], 1.0 mM SA for 5 days. *A*, growth pattern of MEL cells with and without hematin for 2 days following the heme depletion of the cells for 4 days; *B*, heme concentrations of these cells for 2 days. Similar results were obtained in another experiment.

controls when concentrations of SA of either 0.3 or 1.0 mM were present during the first 4 days. When the medium containing SA was replaced on Day 4 with fresh medium supplemented with hematin at a concentration of 2 nmol/ml, the values rose markedly above controls, and they rose moderately above controls when the hematin concentration was 300 pmol/ml.

In Chart 3*A*, the effects on growth of heme depletion and repletion over a 48-hr period are presented. After exposure to 0.1 mM, 0.3 mM, or 0.6 mM SA for 4 days followed by removal of inhibitor and supplementation of the medium with 500 pmol of hematin per ml, there was no growth stimulation seen after a 48-hr period, except for slight stimulation in cells exposed to the lowest concentration of SA (0.1 mM).

The heme concentrations of these cells are seen in Chart 3*B*. Continuous exposure to 0.3 mM SA caused a marked decrease of cellular heme concentration which remained at very low levels throughout the entire experiment. When medium containing SA was replaced with fresh medium not containing SA, cellular heme levels remained well below those of controls for the entire 48-hr period. When SA was removed and the medium was supplemented with hematin (500 pmol/ml), cellular heme concentrations rose to normal levels at 2 hr and exceeded normal levels for the following 46 hr. As seen in Chart 3*A*, this rapid restoration of cellular heme to normal or above normal levels produced little or no growth stimulation over a 48-hr period.

The uptake of hematin by both control and heme-depleted MEL cells over a 2-hr period is presented in Chart 4. In both instances, addition of 500 pmol hematin per ml to the medium caused cellular heme levels to increase rapidly in both cells which were exposed to 0.5 mM SA and control cells. Increases of cellular heme were evident at 5 min, and further increases were seen at 10 or 30 min. It is seen that both control cells and cells depleted of heme by treatment with SA do not manifest significant changes in cellular heme concentrations during the short duration of this study.

The effect of temperature on hematin uptake over a brief period in normal and heme-depleted MEL cells is seen in Chart 5. In both control and heme-depleted cells, heme levels increased at a rapid rate over a 2-min period after exposure to 500 pmol hematin per ml. Increases observed thereafter appear to be at a much slower rate. The rapid initial uptake appears to be similar in both control and heme-depleted cells and is not markedly affected by temperature differences (20°, 37°).

The cellular distribution of exogenously administered hematin is seen in Table 1. MEL cells were depleted of endogenous heme by growing them in the presence of SA, and then the cellular uptake of hematin was measured. Both normal and heme-depleted cells grown in heme-free medium were able to take up hematin. The heme-depleted cells increased their heme content 9-fold and accumulated enough heme to exceed the normal heme levels (50 to 150 pmol heme per mg protein). The proportion of heme in the 20,000 × *g* sediment from heme-supplemented and untreated control MEL cells was similar. When MEL cells were sonicated rather than freeze thawed, the

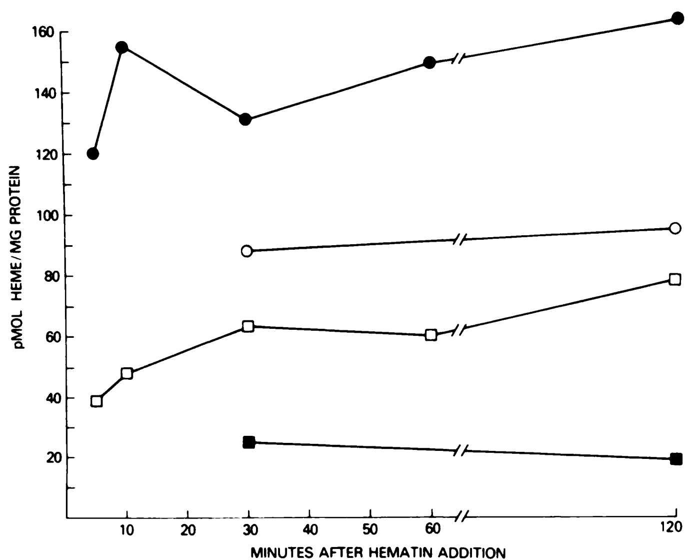

Chart 4. The short-term uptake of hematin by control MEL cells and cells depleted of heme by treatment with SA. MEL cells were grown in 1% gelatin with and without 0.5 mM SA for 4 days. The cells were centrifuged and then incubated in fresh medium for 40 min at 37°. Where indicated, hematin was added and incubated further at 37° for 5 to 120 min. ○, control; ●, control + hematin (500 pmol/ml); □, 0.5 mM SA to Day 4, then hematin (500 pmol/ml); ■, 0.5 mM SA to Day 4. The results were similar in 2 additional experiments.

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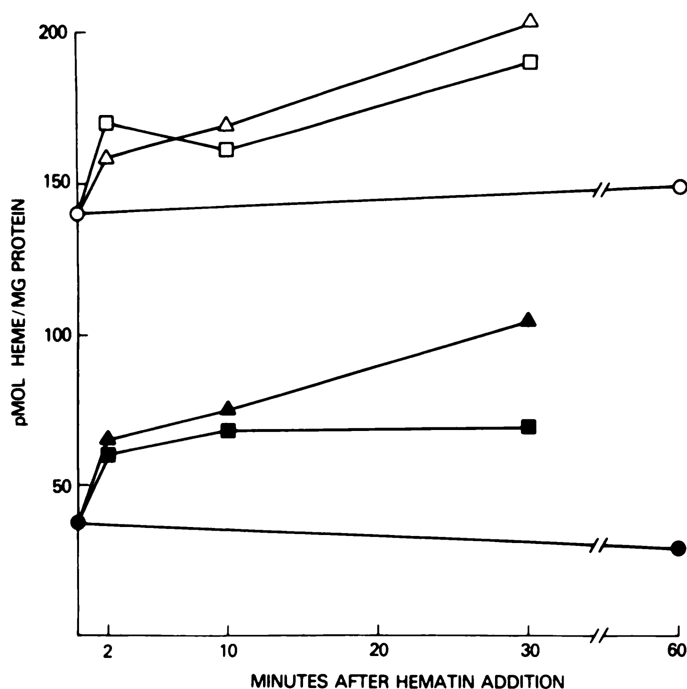

Chart 5. Effect of temperature on the uptake of hematin by MEL cells grown in defined medium with or without SA. MEL cells were grown in Roswell Park Memorial Institute Medium 1640 containing 1% gelatin with and without SA for 4 days. The medium was changed, and the cells were then incubated at either 20° or 37° with hematin. ○, control at 37°; △, control medium for 4 days, then hematin (500 pmol/ml) at 37°; □, control medium for 4 days, then hematin (500 pmol/ml) at 20°; ●, 0.5 mM SA for 4 days, then medium only at 37°; ▲, 0.5 mM SA for 4 days, then hematin (500 pmol/ml) at 37°; ■, 0.5 mM SA for 4 days, then hematin (500 pmol/ml) at 20°. Similar results were seen in 3 additional experiments.

Table 1

Distribution of heme in cellular fractions of freeze-thawed MEL cells treated with and without SA

MEL cells were grown 5 days in Roswell Park Memorial Institute Medium 1640 containing 1% gelatin with and without 1 mM SA. The cells were centrifuged and resuspended in fresh medium without SA. Hematin was then added to the medium, and the incubations were continued for 20 min. Aliquots of cells were harvested by centrifugation, washed 3 times with 10 mM Tris (pH 7.4):0.15 M NaCl, and freeze-thawed 5 times in distilled water. The broken cells were then centrifuged at 20,000 × g for 20 min, and the supernatant and particulate fractions were assayed for their heme content.

SA (mM)	Hematin (pmol/ml)	Whole-cell heme concentration (pmol/mg protein)	% of total heme in particulate fraction
		135 ± 2[a]	88 (82, 93[b])
	1000[c]	394 ± 102[a]	92[b]
1.0		26	78
1.0	1000[c]	235	96

[a] Average values from 3 experiments ± S.E.
[b] Average values from 2 experiments and the data points.
[c] Hematin was incubated with the cells for 20 min at 37°.

proportion of heme found in the particulate fraction of heme-supplemented control cells was 80%, and that in the heme-supplemented heme-depleted cells was 87%. In cells depleted of heme by growth in medium containing SA, there may have been a slight decrease of the proportion of heme present in the 20,000 × g sediment, but this was brought to normal by addition of hematin to the medium.

The effect of exogenous hematin on a contact-inhibited cell line, 3T3, is presented in Table 2. This nonmalignant fibroblastic cell line grows adequately in medium containing 4% FBS. Incubation of these cells with exogenous hematin in a manner similar to that for the MEL cells showed that hematin uptake by 3T3 fibroblasts was not evident when individual comparisons of hematin-treated and control cells were made under individual conditions with 3 methods of harvest. However, a 22% increase in uptake was demonstrated when statistical analysis was applied to multiple samples. Different methods of harvest were attempted to assure that adsorbed hematin was not removed from the cell membrane by the trypsin treatment. To exclude the possibility that the exogenous hematin present in 4% FBS was masking the uptake of hematin in 3T3 cells, MEL cells were similarly grown in 4% FBS and then exposed to hematin at 1 nmol/ml for 30 min. The MEL cells readily took up the added hematin despite the presence of a low level of heme in the medium (data not shown). Growth of 3T3 cells for 6 days in medium containing 2 mM SA was accompanied by a decline of about 30% of cellular heme content. Hematin uptake did not occur in heme-depleted 3T3 cells, and only low levels of hematin were taken up in nondepleted control cells.

Table 2

Uptake of hematin by untreated and SA-treated 3T3 cells

3T3 cells were grown with and without 2 mM SA in McCoy's Medium 5A containing 4% FBS for 4 to 6 days after seeding the cells. When the cells were near confluence, the medium was poured off and replaced with fresh medium without SA but containing hematin as described. The cells were either trypsinized or scraped off the flask, washed 3 times with 10 mM Tris (pH 7.4):0.15 M NaCl, and frozen prior to heme assay.

No. of tests	SA (mM)	Hematin (pmol/ml)	Method of harvest	Heme concentration (pmol/mg protein)[a]
1			Trypsin	74
1		500[b]	Trypsin	67
1		500[b]	Trypsin[c]	76
9			Mechanical	79 ± 3
6		1000[d]	Mechanical	96 ± 2[e]
1		2000[f]	Mechanical	61
2	2.0		Mechanical	56 (51, 62)
2	2.0	1000[d]	Mechanical	47 (43, 52)

[a] Average heme concentration ± S.E. or (range).
[b] Exogenous hematin present for 20 min at 37°.
[c] Cells trypsinized prior to hematin addition.
[d] Exogenous hematin present for 30 min at 37°.
[e] Statistically significant from the untreated control ($p < 0.001$) by Student's *t* test.
[f] Exogenous hematin present for 90 min at 37°.

DISCUSSION

Heme and porphyrins have each been demonstrated to be significant to tumor cells but in different contexts. First is the fact that certain porphyrins are known to exhibit a selective affinity for neoplastic tissues (1, 2, 4, 6–8). Hematoporphyrin showed a high affinity for human malignant neoplasms (6) and tumor cells in rats bearing the ascites hepatoma (7). The hydrophobic porphyrin mesoporphyrin IX accumulated rapidly at hydrophobic loci of leukemia L1210 cells (8). With a more hydrophilic porphyrin, *meso*-tetra(*p*-sulfophenyl)porphine, the porphyrin was accumulated in a tumor cell (HEp-2 cells) by a diffusion process (1). Except for preliminary studies in MEL cells (3, 13), the uptake of the natural metalloporphyrin hemin in tumor cells has not been examined.

The significance of heme in tumor cell growth is indicated by the fact that inhibition of heme biosynthesis in MEL cells by SA caused a decrease of cellular heme content concomitant with inhibition of growth (3, 13, this study). MEL cell growth was not severely restricted until the intracellular heme level was

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depleted to about 0 to 10 pmol/mg protein. In numerous experiments, heme concentrations in untreated MEL cells varied between 30 and 150 pmol/mg protein. Partial depletion of heme down to about 15 pmol/mg protein was insufficient in most cases to restrict cell growth. When cellular heme concentrations declined to the range of 10 to 15 pmol/mg protein, growth inhibition was usually seen and always occurred with concentrations below 10 pmol/mg protein.

Although the effects of hemin on hemoglobin synthesis in MEL cells have been examined by several workers, only preliminary studies have been performed on heme uptake by these cells during normal growth or when heme depleted (13). Some of the biological effects of hemin on MEL cells have been described. Exogenous hemin enhanced hemoglobin synthesis when present with inducer and was detected in the hemoglobin produced by the cells (10). Hemin alone and in the presence of inducer stimulated the production of mRNA for globin (11). Iron accumulation into a ferritin-like complex was increased in the presence of hemin in uninduced MEL cells but was decreased in induced cells (10). Hemin also had a stimulating effect on MEL cell maturation (10). All these experiments show that hemin can readily be taken up by the cell and utilized. Our data show also that hemin can be taken up by the cells and utilized to stimulate growth following depletion of intracellular heme levels and cessation of growth.

The uptake of hematin in MEL cells occurs as a bimodal process and does not appear to be an enzymatic mechanism. As shown in Chart 4, in both untreated and heme-depleted cells, hematin was rapidly taken up from the medium, and then the cellular concentration slowly increased further for the next 28 min. Although the uptake of hematin at 37° after 30 min was somewhat greater than the uptake at 20° in both the untreated and heme-depleted cells, the magnitude of the difference was not characteristic of an enzymatic process. In fact, heme uptake was observed in untreated and heme-depleted cells incubated at 0° in a medium containing neither serum nor gelatin (data not shown). The 2-stage process of heme uptake is seen in both Charts 3 and 4. There is no suggestion of induction of a cellular heme uptake mechanism in heme-depleted cells, since the rate of uptake was about the same in both normal and heme-depleted cells (Charts 2 to 4; Table 1). In a study of mesoporphyrin IX uptake in L1210 cells (8), a bimodal uptake of this hydrophobic porphyrin was observed also. The rapid uptake phase occurred within 1 min in serum-free medium and was more pronounced with increasing doses of mesoporphyrin. The lack of a demonstrable temperature coefficient for hematin uptake in the present study using hematin resembles similar data for mesoporphyrin uptake in L1210 cells (7).

Uptake of hematin was observed in both untreated and heme-depleted MEL cells. Although cellular heme levels increased by a factor of 9 in heme-depleted MEL cells which were exposed to exogenous hematin compared to only a 3-fold increase in MEL cells not depleted of hematin (Table 1), the absolute values of these increases (209 pmol/mg protein in the heme-depleted cells *versus* 259 pmol/mg protein in cells not depleted of heme) were not markedly different. The fact that the absolute uptake of exogenous hematin by MEL cells which were and by others which were not depleted of heme was similar suggests that there is a membrane binding or uptake capacity for hematin in these cells.

A similar attempt was made to examine the potential heme uptake mechanism in a normal fibroblastic cell line, 3T3. Unlike the MEL cells, little heme uptake was observed in 3T3 cells grown in medium containing the minimum concentration of FBS to support growth. In contrast, much higher levels of heme uptake could be demonstrated in MEL cells grown in medium containing 4% FBS than in 3T3 cells grown under similar conditions. This finding negates the possibility that the low level of endogenous hematin present in the medium of 3T3 fibroblasts masked the observation of a potential uptake mechanism in this normal cell. A concentration of 2 mM SA permitted cell growth with a 30% decrease of cellular heme concentration. No evidence of the activation of a heme uptake mechanism was observed in this fibroblastic line when partially depleted of heme.

The fact that measurements of hematin uptake by MEL cells at different temperatures did not suggest an enzymatic mechanism raises the question of whether the apparent hematin uptake may only represent nonspecific adsorption of hematin to the cell membrane of MEL cells. That this is not the case is suggested by not only the above-cited previous studies of the effects of exogenous hematin on hemoglobin synthesis and iron accumulation but also its effect on growth stimulation in heme-depleted MEL cells. A more specific role of hematin uptake is further suggested by the low rate of hematin uptake by fibroblasts as shown in the present studies.

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